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Peptide Inhibitors for Angiotensin I-Converting Enzyme from Thermolysin Digest of Dried Bonito†

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Dried bonito (*Katsuobusi*), a Japanese traditional seasoning made of bonito muscle was hydrolyzed by various proteases and the inhibitory activity of the hydrolyzates for angiotensin I-converting enzyme (ACE) [EC 3.4.15.1] was measured. Among the digests, thermolysin digest showed the most potent inhibitory activity. Eight inhibitory peptides were isolated from the digest using HPLC. The amino acid sequences of inhibitory peptides were Ile-Lys-Pro-Leu-Asn-Tyr, Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly, Ile-Trp-His-His-Thr, Ala-Leu-Pro-His-Ala, Phe-Gln-Pro, Leu-Lys-Pro-Asn-Met, Ile-Tyr, and Asp-Tyr-Gly-Leu-Tyr-Pro. By searching for the sequence homology in many proteins, four of them were found in the primary structure of actin. Asp-Met-Ile-Pro-Ala-Gln-Lys was obtained from the boiling water extract of dried bonito and this peptide was found in the primary structure of creatine kinase. Fragments of these peptides were prepared by further enzymatic digestion or chemical synthesis and their ACE-inhibitory activities were measured. Among them, Ile-Lys-Pro, Ile-Trp, Leu-Lys-Pro, and Leu-Tyr-Pro had higher inhibitory activity than their parental peptides. Ile-Lys-Pro suppressed the hypertensive activity of angiotensin I.

The angiotensin I-converting enzyme (ACE) catalyzes the formation of angiotensin II, a strong pressor, from angiotensin I and inactivates bradykinin, which has depressor action. Inhibitors of this enzyme show antihypertensive activity.[1] Commercial antihypertensive drugs such as captopril and enalapril are very potent ACE inhibitors. Recently, ACE-inhibitory peptides derived from casein,[2-4] fish muscle,[5,6] and other food proteins[7,8] were isolated. Although the inhibitory activities of these food-derived peptides are weaker than those of drugs such as captopril and cnarapril, some of them have been shown to be effective in lowering blood pressure of spontaneously hypertensive rats (SHR) after oral administration.[6,9] Some of these peptides, however, have a bitter taste and those from fish protein have a fishly odor. We found that protease digests of dried bonito had ACE-inhibitory activity in addition to a good taste. We also found that the thermolysin digest had the most potent inhibitory activity among the digests tested. We tried to isolate ACE-inhibitory peptides from the thermolysin digest of dried bonito muscle.

Materials and Methods

Materials. Pepsin (porcine stomach mucosa), trypsin (bovine pancreas, Type I), chymotrypsin (bovine pancreas, Type VII), and rabbit lung acetone powder were purchased from Sigma Chemical Co. Thermolysin and hippuryl-histidyl-leucine (HHL) were purchased from the Peptide Institute Inc. *t*-Butyloxycarbonyl amino acids were purchased from Watanabe Chemical Ind., Ltd.

Digestion of dried bonito. Five grams of dried bonito muscle was suspended in 45 ml of distilled water and homogenized with a Polytron (Kinematica GmbH PT 10/35, Switzerland) for 1 min. The homogenate was boiled for 10 min. The boiled homogenate was digested by various proteases (pepsin, chymotrypsin, trypsin, and thermolysin). Enzymatic digestion was done in the presence of 880 μg/ml of proteases at 37°C for 3 hr. Before the pepsin digestion, the homogenate was adjusted to pH 2 with hydrochloric acid. Digestions by other proteases were done at pH 7.5. The digests were boiled for 10 min to inactivate the enzymes. The pepsin digest was adjusted to pH 7 with sodium hydroxide before boiling. The supernatants were recovered after centrifugation (supernatant A). Supernatant was also obtained without enzymatic digestion (supernatant B). Supernatant B was also hydrolyzed by various proteases as described above and the centrifugal supernatants were obtained (supernatant C).

Measurement of ACE-inhibitory activity. ACE-Inhibitory activities was measured using HHL as a substrate and extract of rabbit lung acetone powder by the method of Cushman and Cheung.[10] Each assay mixture contained the following components at the indicated final concentration: potassium phosphate buffer, 100 mM; sodium chloride, 300 mM; HHL, 5 mM; and enzyme 3 mU per 250 μl of assay volume (1 unit of ACE activity is defined as the amount catalyzing the formation of 1 μmol of hippuric acid from HHL in 1 min at 37°C). The IC_{50} value is the concentration of 50% ACE inhibition in the reaction mixture. Total activity was defined as the volume of the solution obtained from 1 g of dried bonito of which the concentration is IC_{50}.

Purification of peptides from thermolysin digest of dried bonito. The thermolysin digest (supernatant A) was put on a octadecyl silica (ODS) column (YMC-Pack ODS-AQ, SH-343-5, 20 × 250 mm, YMC Co., Ltd.). The column was developed at a flow rate of 10 ml/min by a linear gradient of acetonitrile (1—41%/40 min) containing 0.1% trifluoroacetic acid. Individual fractions were dried with a centrifugal concentrator and their inhibitory activities were measured. The active fractions were purified on a phenyl silica (Ph) column (Cosmosil 5Ph, 4.6 × 250 mm, Nacalai Tesque Inc.), which was developed at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0—40%/40 min) containing 0.1% trifluoroacetic acid. The active peaks from the phenyl column were purified on a cyanopropyl silica (CN) column (Cosmosil 5CN R,

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Table I. ACE Inhibition by Enzymatic Hydrolyzate of Dried Bonito

	Supernatant A			Supernatant C
	IC_{50} (μg/ml)	Solubilized peptide (%)	Total activity (liter*/g dried bonito)	IC_{50} (μg/ml)
Thermolysin	29	55.3	19.1	120
Trypsin	161	34.9	2.17	254
Chymotrypsin	117	20.1	1.72	215
Pepsin	47	67.0	14.3	142
Trypsin + Chymotrypsin	175	15.9	0.909	362
Pepsin→Trypsin	65	75.5	11.6	222
Pepsin→Chymotrypsin	41	77.8	19.0	307
Pepsin→Trypsin + Chymotrypsin	38	60.7	16.0	123

* Volume of the solution of which concentration is IC_{50}.
Supernatant B, IC_{50} = 174 μg/ml; solubilized peptide, 5.61%; total activity, 0.324 liter/g.

4.6 × 250 mm), which was developed as described above. When the obtained peptide was not pure after the three HPLC steps, the peptide was further purified on a ODS column (Cosmosil 5C18, 4.6 × 150 mm) which was developed at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0—40%/40 min) containing 10 mM phosphate buffer (pH 7.0).

Amino acid sequence analysis. The amino acid sequence of the purified peptide was analyzed by a protein sequencer (477A, Applied Biosystems Inc.).

Synthesis of peptides. Peptides were synthesized by a solid phase method with a peptide synthesizer (Biosearch SAM TWO). *t*-Butyloxycarbonyl amino acids were successively coupled in the presence of *N,N'*-diisopropylcarbodiimide. Peptides were deprotected by the anisole/hydrogen fluoride method and purified on HPLC.

Measurement of blood pressure. A Wistar male rat weighing from 200 to 300 g was anesthetized by intraperitoneal administration of urethane at 1—1.5 g/kg. The carotid artery blood pressure was measured with a pressure transducer (Spectramed Medical Products (s) Pte., Ltd.). Peptide was dissolved in saline and injected into the femoral vein using a catheter.

Results

1. *Digestion of dried bonito by various proteases*

Dried bonito was hydrolyzed by pepsin, trypsin, chymotrypsin, and thermolysin independently or in combination (Table I). In any protease studied, the inhibitory activity of supernatant A was higher than that of supernatant C. Especially, supernatant A of thermolysin hydrolyzate showed the most potent inhibitory activity (IC_{50} = 29 μg/ml). Total activity of supernatant A for the case of hydrolysis by thermolysin or both pepsin and chymotrypsin was about sixty times higher than that of supernatant B (boiling water extract of dried bonito). Supernatant B showed an IC_{50} value of 174 μg/ml. Inhibitory activity increased slightly when the supernatant B was hydrolyzed by thermolysin or pepsin or the combination of pepsin, trypsin, and chymotrypsin. In other cases, inhibitory activities of supernatant C were weaker than that of supernatant A. These results indicate that the inhibitory active peptides in supernatant A are mainly released by the digestion of insoluble matter in the homogenate. The thermolysin digest had the most potent inhibitory activity. Thermolysin, as well as the chymotrypsin or trypsin digest had no bitter taste but had a good taste characteristic of dried bonito. On the other hand, the pepsin digest had a bitter taste. The ACE-inhibitory activity of the thermolysin digest did not change after further digestion by pepsin, trypsin, and chymotrypsin (Table II). We then tried to isolate the inhibitory peptides from the thermolysin digest.

Table II. Effects of Further Treatments of the Thermolysin Digest of Dried Bonito by Gastrointestinal Proteases

	IC_{50} (μg/ml)	Solubilized peptide (%)	Total activity (liter*/g dried bonito)
None	29	55.3	19.1
Pepsin	22	57.3	26.0
Pepsin→Trypsin	26	56.4	21.7
Pepsin→Chymotrypsin	29	56.1	19.3
Pepsin→Trypsin + Chymotrypsin	26	56.1	21.6

* Volume of the solution of which concentration is IC_{50}.

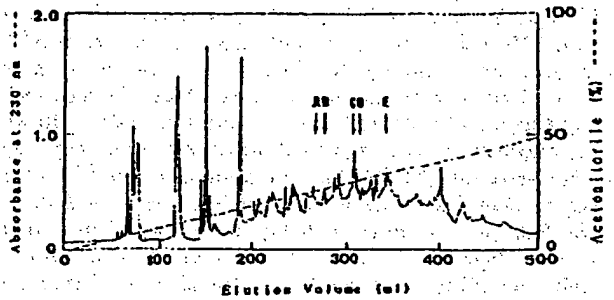

Fig. 1. HPLC of Thermolysin-digest of Dried Bonito on ODS Column.
Individual fractions corresponding to 2 mg of the original extract were added to the reaction mixture. Arrows indicate active fractions. Chromatography was done as described in Materials and Methods.

2. *Isolation of ACE-inhibitory peptides*

About eighty peaks were detected when the thermolysin digest was fractionated on the ODS column (Fig. 1). Inhibitory fractions (A, B, C, D, E) were eluted at 26—33% acetonitrile (Fig. 1). The active fractions were further purified on phenyl and cyanopropyl columns, successively. Fraction E was further purified on the ODS column (Fig. 2, Table III). Eight ACE-inhibitory peptides were isolated. Using the protein sequencer, primary structures of the individual peptides were identified. Among

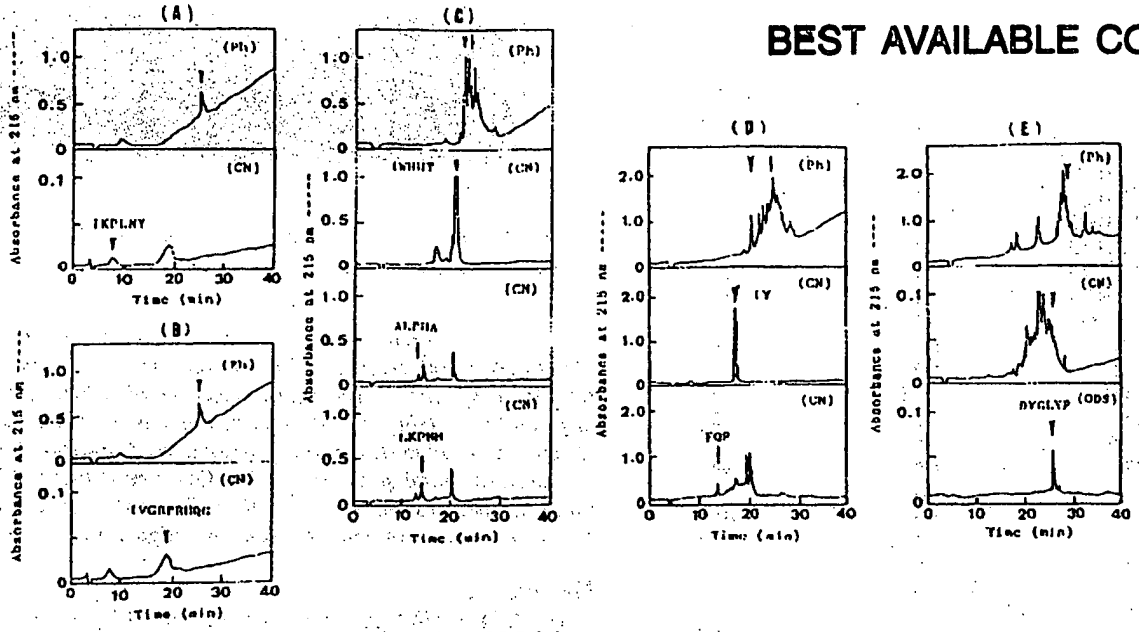

Fig. 2. Purification of Active Peaks from ODS Column.

Peaks A—D were purified on phenyl (Ph) and cyanopropyl (CN) columns. Peak E was further purified on an ODS column.

Table III. Summary of Purification of Active Peaks

Peaks	CH_3CN Concentration of Elution (%) ODS (0.1% TFA)	Ph (0.1% TFA)	CN (0.1% TFA)	ODS (10 mM KPi)	Peptides
Thermolysin digest					
A	26	24	8		IKPLNY
B	27	24	19		IVGRPRHQG
C	30	22	20		IWHHT
		23.5	12		ALPHA
			13		LKPNM
D	31	20	17		IY
		24	14		FQP
E	33	28	26	25	DYGLYP
Boiling water extract					
	23	23	13		DMIPAQK

Table IV. ACE-Inhibitory Peptides Derived from Dried Bonito

Peptides	ODS Peak	IC_{50} (μM)	Origin
Thermolysin digest			
IKPLNY	A	43	(Myosin)
IVGRPRHQG	B	6.2	Actin
IWHHT	C	5.1	Actin
ALPHA	C	10	Actin
LKPNM	C	17	
IY	D	3.7	
FQP	D	12	Actin
DYGLYP	E	62	(Fibronectin)
Boiling water extract			
DMIPAQK		45	Creatine kinase

them, Ile-Tyr had the most potent inhibitory activity and showed an IC_{50} of 3.7 μM. Ile-Trp-His-His-Thr and Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly also had an IC_{50} of 5.1 and 6.2 μM, respectively. Ala-Leu-Pro-His-Ala, Phe-Gln-Pro, Leu-Lys-Pro-Asn-Met, Ile-Lys-Pro-Leu-Asn-Tyr, and Asp-Tyr-Gly-Leu-Tyr-Pro showed IC_{50} values of 10, 12, 17, 43, and 62 μM, respectively (Table IV).

Among them, Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly, Ile-Trp-His-His-Thr, Ala-Leu-Pro-His-Ala, and Phe-Gln-Pro were found in the primary structure of chicken actin (Fig. 3). Though the primary structure of bonito actin is unknown, these four peptides may be released from bonito actin. A sequence homologous to Ile-Lys-Pro-Leu-Asn-Tyr, Ile-Lys-Glu-Leu-Asn-Tyr, was found in the primary structure of rat myosin. A sequence homologous to Asp-Tyr-Gly-Leu-Tyr-Pro, Asp-Tyr-His-Leu-Tyr-Pro, was also found in the primary structure of human fibronectin.

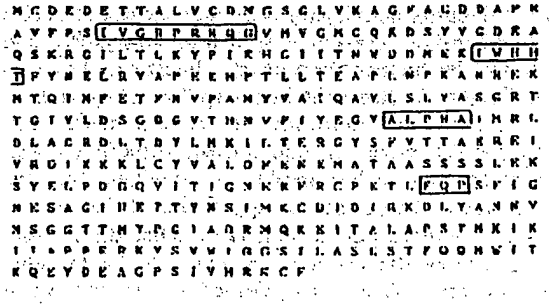

Fig. 3. Primary Structure of Actin of Chicken Muscle.

ACE-inhibitory peptides derived from dried bonito are boxed.

An ACE-inhibitory peptide was also isolated from supernatant B by a three-steps purification using HPLC (data not shown). The sequence of the peptide was Asp-Met-Ile-Pro-Ala-Gln-Lys. This peptide was found in the C-terminal of creatine kinase. This peptide was the major ACE-inhibitory peptide of the boiling water extract.

Table V. ACE-Inhibitory Activities of Synthetic Derivative Peptides

Peptides	IC_{50} (μM)	IC_{50} (μg/ml)
IKPLNY	43	32
IKP	1.7	0.61
LNY	81	33
IVGRPRHQG	6.2	6.3
VGRPRHQG	5.4	4.9
GRPRHQG	34	27
RPRHQG	22	16
PRHQG	55	33
RHQG	330	160
HQG	745	250
IVGRPR	300	210
GRPR	470	230
IWHHTF	2.5	2.1
IWHHT	5.1	3.5
WHHTF	46	33
WHHT	110	64
HHTF	84	45
HHT	800	310
IW	2.0	0.63
ALPHA	10	5.1
ALP	240	72
LKPNM	17	10
LKP	1.6	0.57
DYGLYP	62	45
YGLYP	260	160
GLYP	190	85
LYP	6.6	2.6
YP	890	250
DYG	2700	950
DY	100	30
YG	1100	260
DMIPAQK	45	36
MIPAQK	300	210
IPAQK	260	140
PAQK	100	44
AQK	1800	620
QK	885	240

3. *Synthesis of ACE-inhibitory peptides and their fragments*

Inhibitory peptides from the thermolysin digest of dried bonito were synthesized. Various fragments were also obtained by chemical synthesis or enzymatic hydrolysis, and their inhibitory activity for ACE was measured (Table V). Ile-Lys-Pro-Leu-Asn-Tyr is split into Ile-Lys-Pro and Leu-Asn-Tyr by further thermolysin digestion. Asp-Tyr-Gly-Leu-Tyr-Pro is also split into Asp-Tyr-Gly and Leu-Tyr-Pro by the same conditions. The IC_{50} of Ile-Lys-Pro and Leu-Tyr-Pro were 1.7 and 6.6 μM, respectively and these peptides had higher inhibitory activities than the original peptides. These results suggest that the inhibitory activity may be more potent with the further thermolysin digestion. Among the fragment peptides of Ile-Trp-His-His-Thr, Ile-Trp, which was released by chymotrypsin digestion, showed higher activity (IC_{50} = 2.0 μM) than the parental peptide. Therefore, this peptide will be released by gastrointestinal digestion when the thermolysin digest is ingested. The IC_{50} of Leu-Lys-Pro, a synthetic fragment of Leu-Lys-Pro-Asn-Met, was 1.6 μM. Among the synthetic peptides, Leu-Tyr-Pro and Val-Gly-Arg-Pro-Arg-His-Gln-Gly showed higher activity than the original peptides.

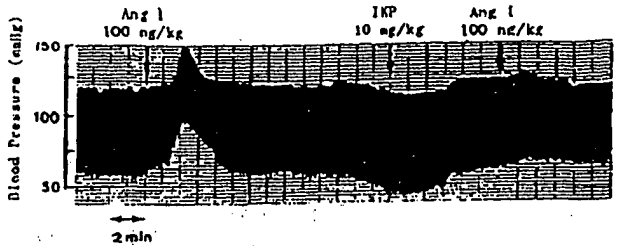

Fig. 4. Effects of Ile-Lys-Pro on Blood Pressure of a Normotensive Rat.
Angiotensin I and the peptide were given intravenously.

4. *Antihypertensive activities of ACE-inhibitory peptides*

Systolic blood pressure of normotensive rat is elevated by 20 mmHg after intravenous injection of 100 ng/kg of angiotensin I (Fig. 4). Ile-Lys-Pro given intravenously at a dose of 10 mg/kg before angiotensin I inhibited the elevation of blood pressure. Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly also showed a similar effect. The antihypertensive effects of other peptides are under investigation.

Discussion

The ACE-inhibitory activity was estimated by measuring the release of hippuric acid from HHL. It should be noted that not only inhibitors but also substrates are detected in this system. The ACE-inhibitory activities of the thermolysin digest of dried bonito is the most potent among the various protease digests. We then isolated and identified eight ACE-inhibitory peptides from this digest. From the substrate specificity of thermolysin, the obtained peptide has Ile, Leu, Phe, or Ala at the amino terminus. Four inhibitory peptides were found in the primary structure of actin. Among them, Ile-Val-Gly-Arg-Pro-Arg-His-Gln-Gly was also obtained from the thermolysin digest of chicken muscle (unpublished data).

The ACE-inhibitory activity of synthetic dipeptides including Ile-Tyr has been reported.[11] Ile-Tyr is the first example of a dipeptidic ACE inhibitor derived from fish. Generally, di- or tripeptides are easily absorbed in the intestine. In fact, the thermolysin digest of dried bonito showed antihypertensive activity by oral administration in SHR or stroke-prone SHR (unpublished data). On the other hand, the pepsin digest or the pepsin-trypsin-chymotrypsin digest did not show antihypertensive activity under the same conditions. Therefore, it is effective to hydrolyze dried bonito by microbial proteases such as thermolysin before we eat them.

Kohama *et al.* isolated an ACE-inhibitory peptide from an acid extract of tuna.[5] Suetsuna *et al.* isolated ACE-inhibitory peptides from a pepsin digest of sardines.[6] The ACE-inhibitory peptide probably derived from sardine actin has been reported during the preparation of this manuscript.[12] Sugiyama *et al.* reported antihypertensive activities of the alkaline protease digest of sardines.[13]

Usually enzymatic digests of fishes have a fishy odor. We found the pepsin digest of sardine to have a bitter taste. The thermolysin digest of dried bonito dose not have a bitter taste or fishy odor but has a good taste. A boiling water extract of dried bonito muscle is a traditional flavor enhancer in Japan. In the process of its production, the muscle is cleared of fats, boiled, and dried. A good

taste called "Umami" is produced during such a process. "Umami" is mainly derived from inosinic acid. Amino acids and peptides, which are produced by proteases, also may contribute to this good taste. Recently, a new concept called "functionalities of foods" has been proposed[14]; foods have nutritional function (primary function), sensory function (secondary function), and physiological function (tertiary function). The thermolysin digest of dried bonito has excellent primary, secondary, and tertiary functions, and is expected to be a physiologically functional foodstuff having antihypertensive effects.

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